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SECRETORY PROTEIN TRAFFICKING AND ORGANELLE DYNAMICS IN LIVING CELLS[1]

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■ **Abstract** Green fluorescent protein chimerae acting as reporters for protein localization and trafficking within the secretory membrane system of living cells have been used in a wide variety of applications, including time-lapse imaging, double-labeling, energy transfer, quantitation, and photobleaching experiments. Results from this work are clarifying the steps involved in the formation, translocation, and fusion of transport intermediates; the organization and biogenesis of organelles; and the mechanisms of protein retention, sorting, and recycling in the secretory pathway. In so doing, they are broadening our thinking about the temporal and spatial relationships among secretory organelles and the membrane trafficking pathways that operate between them.

CONTENTS

INTRODUCTION

The secretory membrane system allows cells to regulate delivery of newly synthesized proteins, carbohydrates, and lipids to the cell surface—a necessity for growth and homeostasis. The system is made up of distinct organelles [including the endoplasmic reticulum (ER), Golgi complex, and plasma membrane] and tubulovesicular transport intermediates that mediate intracellular membrane transport between them (Figure 1). Membrane traffic within this system flows along highly organized directional routes. Secretory cargo is synthesized and assembled in the ER and then transported to the Golgi complex for further processing and maturation. Upon arrival at the *trans* Golgi network (TGN), it is sorted and packaged into post-Golgi carriers that move through the cytoplasm to fuse with the cell surface. This directional membrane flow is balanced by retrieval pathways that bring membrane and selected proteins back to the compartment of origin.

Classical morphological studies of fixed cells have provided the framework for understanding the organization and distribution of structures that make up the secretory membrane system (Palade 1975), and genetic and biochemical studies have given insight into the molecular machinery for its regulation (Schekman & Orci 1996, Rothman & Wieland 1996). Studies using green fluorescent protein (GFP) technology are now addressing the dynamics of this system (reviewed in Lippincott-Schwartz 1998), providing direct observations of intracellular structures whose life history and pathways have previously only been deduced. Time-lapse imaging of GFP-tagged secretory cargo molecules, for example, have provided the first temporal descriptions of the origin, pathway, and fate of secretory transport intermediates. Quantitation of fluorescence from these chimerae has allowed measurement of the number of molecules being transported in small structures (e.g. vesicles and tubules) and the kinetic parameters of membrane traffic to be described in an individual cell. The availability of GFP variants with differing excitation/emission spectra has enabled double labeling to simultaneously visualize two sets of proteins within cells. Photobleaching studies using GFP-tagged organelle markers have been used to study protein diffusional mobility within specific organelles, providing insight into the mechanisms of protein retention within these compartments and the extent of protein cycling between them. Moreover, high-resolution imaging of organelles labeled with GFP fusion proteins has clarified the morphological transformations of these compartments during mitosis and after cellular perturbations. Results from these studies, as discussed in this review,

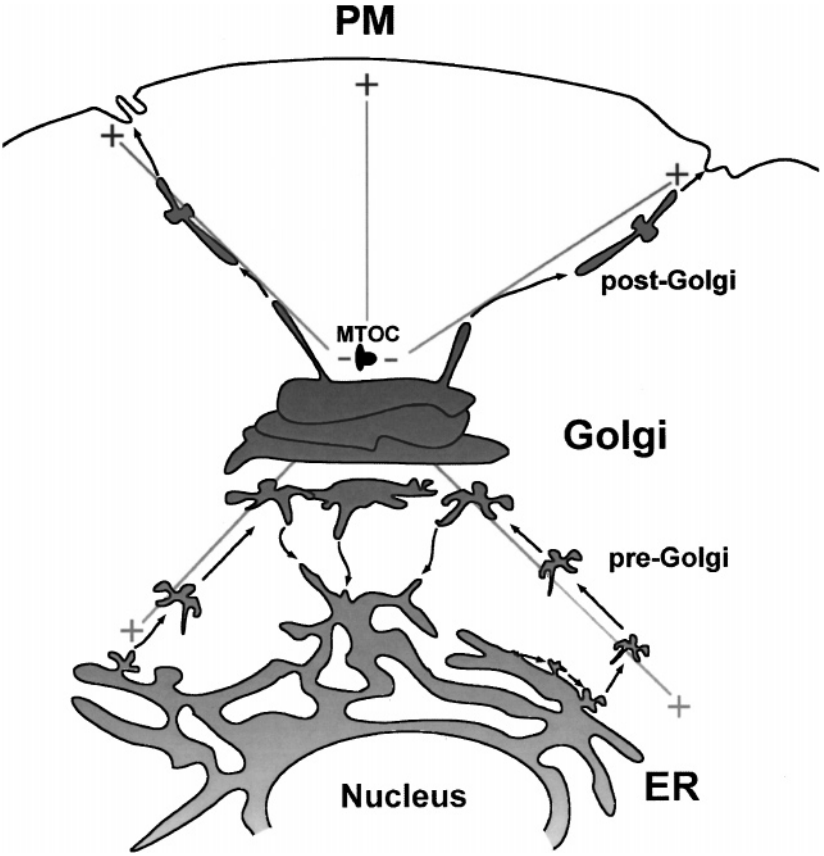

Figure 1 Schematic representation of the secretory pathway. Secretory cargo destined to be secreted or to arrive at the plasma membrane (PM) leaves the ER via distinct exit sites that bud and translocate as tubular-vesicular structures (pre-Golgi) toward the (−) end of microtubules. Here they merge with Golgi membranes (Golgi), which in many mammalian cells are located near the microtubule organizing center (MTOC). After passing through the Golgi complex, secretory cargo is packaged into post-Golgi transport intermediates (post-Golgi), which translocate plus-end directed along microtubules to the plasma membrane.

are providing important new insights into secretory membrane trafficking and the organization and biogenesis of secretory organelles.

ER ORGANIZATION, CONTINUITY, AND RETENTION

The ER is the starting point of the secretory pathway. It is the largest intracellular compartment, with an extensive array of interconnecting membrane tubules and cisternae that extend throughout the cell including the nuclear envelope. ER

membranes are physiologically active, interact with the cytoskeleton, and contain differentiated domains specialized for distinct functions. These functions include protein folding, assembly and degradation, lipid metabolism, detoxification, compartmentalization of the nucleus, regulation of calcium ion gradients, and membrane transport. Recent studies using GFP chimerae have provided insight into the basic physiochemical properties of the ER and the mechanisms underlying ER protein retention and sorting.

Structural Continuity and Lumenal Properties of the ER

ER membranes are differentiated into rough and smooth regions (RER and SER, respectively), depending on whether ribosomes are associated with their cytoplasmic surfaces. Whereas the RER is the site of cotranslational membrane insertion of proteins, the SER is thought to be the site of lipid biosynthesis, detoxification, and calcium regulation. An important question, therefore, is whether these different ER domains represent stably organized subcompartments or are part of a single continuous ER system.

Photobleaching techniques, including fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), using GFP-tagged proteins localized in the ER have helped to address this question. In FRAP, fluorescent molecules in a small region of the cell are irreversibly photobleached by high laser power, and recovery of fluorescence by diffusional exchange of bleached for unbleached molecules is monitored by imaging at low-level illumination (Edidin 1994). The rate and extent to which fluorescence recovers provide a measure of the mobility of the tagged protein and the fraction of the pool of protein that is mobile. In FLIP, fluorescence in one area of the cell is repetitively photobleached while images of the entire cell are collected (Cole et al 1996b). If the area being bleached exchanges GFP-tagged proteins with another region of the cell, loss of fluorescence will occur from both places, allowing assessment of a protein's mobility within either a single organelle or the whole cell.

FRAP studies of GFP-tagged lumenal and membrane ER markers have revealed that these proteins rapidly diffuse throughout the ER, with no regions restricted for diffusional exchange of these molecules (Cole et al 1996b, Subramanian & Meyer 1997, Dayel et al 1999, Nehls et al 2000). When a small area of ER membranes expressing these chimerae is repetitively photobleached in FLIP experiments, complete loss of ER-associated fluorescence from the photobleached cell is observed (including the nuclear envelope) (Cole et al 1996b, Dayel et al 1999). These results indicate that the membranes and lumenal spaces of the ER are normally continuous throughout the cell and that RER and SER form an interconnected membrane system.

The diffusion coefficient, D, of numerous GFP-tagged membrane proteins localized to the ER (including signal recognition particle receptor, lamin B receptor,

galactosyltransferase, mannosidase II, MHC class I, and KDEL receptor) has been measured using FRAP (Cole et al 1996b, Ellenberg et al 1997, Marguet et al 1999, Nehls et al 2000). All these ER proteins have been found to be highly mobile (D ranging from 0.2 to 0.5 $\mu m^2/s$) with little or no immobile fractions. These D values are near the theoretical limit for protein diffusion in a bilayer (Hughes et al 1982), indicating that the ER proteins are not impeded in their lateral mobility. By contrast, the D values for many proteins embedded in the plasma membrane are considerably lower (Edidin 1994), suggesting that there are often constraints to protein diffusion at the cell surface (possibly because of interactions with the extracellular matrix or with the peripheral cytoskeleton). Lower D values relative to other ER proteins have been measured for GFP-tagged cytochrome P450 and TAP (Szczesna-Skorupa et al 1998, Marguet et al 1999). The fact that TAP complexes, which participate in peptide loading of MHC class I in the ER, consist of hundreds of molecules in complexes estimated to be 600–1000 Å in diameter (Howard 1995) is likely to underlie their lower D because protein diffusion is proportional to the logarithm of the radius of the diffusing molecule (Edidin 1994).

The ER lumen is a major calcium storage site in the cell, with calcium release from ER stores important for many cellular responses. FRAP studies using GFP-tagged lumenal markers have shown that conditions that cause an increase in cytosolic calcium cause structural changes in the ER, including inhibition of lumenal marker diffusion and membrane fragmentation (Subramanian & Meyer 1997). A similar disruption in ER structure has been shown to occur transiently during fertilization of sea urchin eggs (when cytoplasmic calcium levels rise), also using GFP FRAP techniques (Terasaki et al 1996). Understanding the role that calcium plays in these changes in ER structure will be important for studying other physiological processes involving the spatiotemporal control of calcium signaling including apoptosis and induction of gene expression.

Many of the resident proteins of the ER (including protein disulfide isomerase, GRP 94 and GRP 74) exist in very high concentrations in the ER lumen and are thought to form a proteinaceous network with a gel-like consistency (Koch 1987). By applying FRAP to signal sequence-tagged GFP (Dayel et al 1999), a noninvasive ER probe, diffusion of the chimera was found to be 9- to 18-fold slower than diffusion of GFP in water and 3- to 6-fold slower than GFP in the cytoplasm. This confirms that the ER lumen is a highly viscous environment.

Protein Folding, Assembly, and Retention in the ER

Upon post- or cotranslational insertion into ER membranes, newly synthesized proteins encounter lumenal chaperones (including BiP, calnexin, calreticulin, and protein disulfide isomerase) whose role is to facilitate folding reactions necessary for protein maturation and oligomerization (Helenius et al 1992). Because

incorrectly folded and assembled proteins are retained in or degraded from this compartment, the ER plays an important quality control role in protein transport into the secretory pathway (Hammond & Helenius 1995). Little is known regarding the dynamic properties of the interactions between ER chaperones and newly synthesized membrane proteins, and how they result in protein retention, degradation, or export from the ER. Studies using GFP chimerae are beginning to address these issues.

One explanation for why some misfolded proteins are retained in the ER is that they are immobilized through extensive interactions with chaperones and are unable to move to ER exit sites (de Silva et al 1990). To test this possibility, the diffusional mobility of misfolded and correctly folded forms of the temperature-sensitive variant (ts045) of vesicular stomatitis virus G protein tagged with GFP (VSVG-GFP) were compared in FRAP experiments (Nehls et al 2000). ts045-VSVG protein reversibly misfolds and is retained in the ER at 40°C, but upon temperature shift to 32°C it correctly folds and is transported out of the ER into the secretory pathway. This property is unaffected by association with a GFP moiety (Presley et al 1997, Scales et al 1997). No difference was observed in the diffusional mobility or immobile fraction between misfolded VSVG-GFP at 40°C and correctly folded VSVG-GFP at 32°C in cells treated with brefeldin A (BFA), which retains proteins in the ER (Lippincott-Schwartz et al 1989); in either case VSVG-GFP diffused at the theoretical limit for protein in a bilayer (see Figure 2). This indicates that misfolded VSVG complexes are not retained in the ER as a result of forming an immobilized aggregate or being tethered to a meshwork. Complexes of misfolded VSVG proteins at 40°C (including VSVG-BiP and VSVG-calnexin) (de Silva et al 1993) observed in biochemical studies are, therefore, either too small to affect the diffusional mobility of VSVG or highly dynamic.

To test whether misfolded VSVG is retained in the ER by constant retrieval from post-ER compartments, FLIP experiments were performed with VSVG-GFP at 40°C (Nehls et al 2000). Virtually all VSVG-GFP fluorescence in cells at 40°C could be eliminated upon repetitive photobleaching of a small region of the ER with no patches of fluorescence remaining, in contrast to FLIP results with KDELR-GFP (a protein that rapidly cycles between ER and Golgi) where fluorescent patches (corresponding to post-ER structures) remained. This suggests that misfolded VSVG complexes are retained in the ER by failing to be concentrated at ER exit sites rather than by continuous retrieval from a post-ER compartment.

Conditions that impede the dissociation of VSVG from chaperones, including ATP depletion and tunicamycin treatment (Leavitt et al 1977, Braakman et al 1992), were found to cause significant immobilization of VSVG-GFP in the ER (Nehls et al 2000) (Figure 2). This raises the possibility that when interactions between misfolded proteins and ER chaperones become irreversible and extensive, the diffusional mobility of proteins in the ER becomes restricted, perhaps through the formation of an extensive ER matrix. Consistent with this, in cells depleted of ATP the diffusional mobility of signal sequence-tagged GFP is slowed (Nehls et al

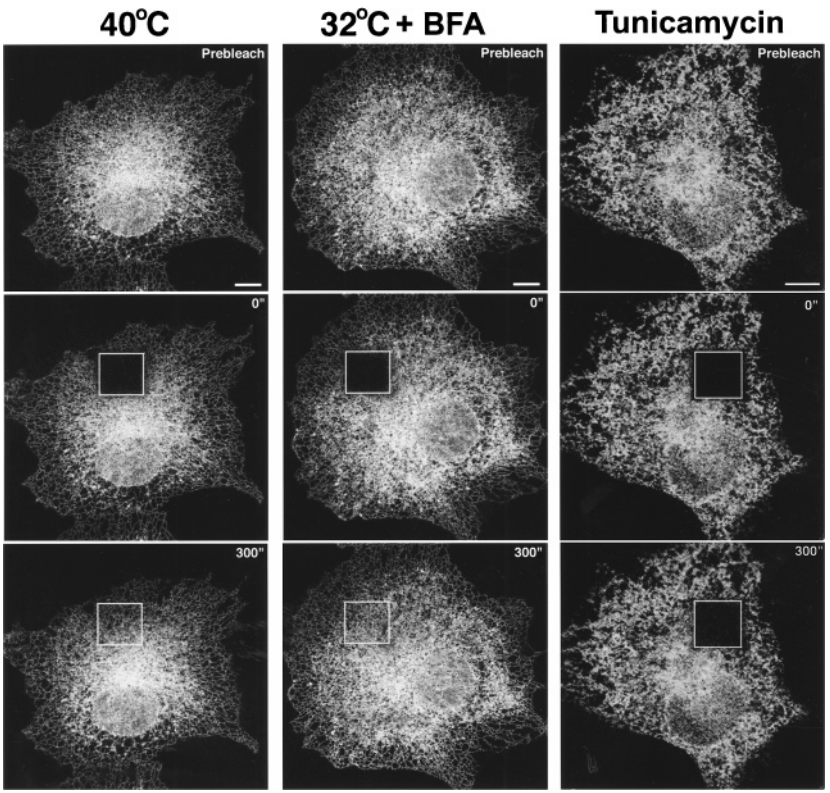

Figure 2 FRAP of VSVG-GFP: misfolded in the ER at 40°C; correctly folded at 32°C in cells treated with BFA; misfolded in the ER in cells treated with tunicamycin. Bar = 10 μm. Note the rapid recovery of fluorescence into the bleached box in cells incubated at 40°C and at 32°C with BFA, but the lack of recovery in cells treated with tunicamycin. This suggests that misfolded VSVG-GFP complexes are highly mobile at 40°C but immobilized after tunicamycin treatment (for details see Nehls et al 2000).

2000), suggesting that the ER lumen alters its overall viscosity when chaperone-protein interactions are increased.

The ability to probe the diffusional characteristics of GFP-tagged proteins in the ER using photobleaching techniques and to visualize ER dynamics using time-lapse imaging has enormous potential for further clarification of the mechanisms underlying protein folding, maturation, and degradation processes associated with the ER. These techniques can also be used to study other ER characteristics, including interactions of protein complexes containing peptide transporters (Marguet et al 1999), the protein translocon (Greenfield & High 1999), and smooth ER domains where lipid is synthesized and metabolized (Náray-Fejes-Tóth & Fejes-Tóth 1996, Ilgoutz et al 1999).

ER-TO-GOLGI TRANSPORT

ER Exit Sites

After proper folding and oligomer assembly, newly synthesized proteins destined for the secretory pathway are selectively separated from ER resident proteins. This occurs at ER exit sites, also termed the transitional ER, which are scattered over the surface of the ER. These exist as highly organized membrane domains (1–2 μm in diameter) adjacent to elaborate tubular clusters called pre-Golgi intermediates, VTCs (vesicular-tubular clusters), or ERGIC (ER-Golgi-intermediate compartment) (Saraste & Kuismanen 1992, Bannykh et al 1996, Hauri et al 2000). ER exit sites observed at the electron microscope level usually contain multiple budding profiles, possibly representing budding vesicles that subsequently fuse locally to form pre-Golgi transport intermediates. Because the membranes at ER exit sites sometimes show direct continuity with the tubule clusters of pre-Golgi intermediates (Clermont et al 1994, Krijnse-Locker et al 1994, Stinchcombe et al 1995, Hermo & Smith 1998), an alternative possibility is that the budding profiles represent ER exit sites transforming directly into pre-Golgi intermediates by fission/fusion without the need for small 50-nm vesicle intermediates.

Secretory cargo is actively sorted and partioned into ER exit sites, so that these domains have an increased level of organization of protein and lipid compared with surrounding ER membranes. This is accomplished by the activity of Sar1 (a ras-related GTPase) and the cytosolic coat protein complex known as COPII (reviewed in Schekman & Orci 1996). COPII assembly onto membranes at ER exit sites initiates with Sec12-mediated nucleotide exchange of GTP (in place of GDP) onto Sar1, with cargo molecules or putative cargo receptors potentially triggering this exchange. Sec23/24 and Sec13/31 heterodimers are sequentially assembled onto the cytoplasmic leaflet of the activated ER membrane to form a COPII-coated bud. This bud is generally thought to then transform into a vesicle although, as suggested above, it may not immediately sever, allowing tubule formation that could cluster with other adjacent COPII-derived tubules. Mutants of COPII components or Sar1 result in a block in protein exit from the ER, demonstrating that assembly of the COPII coat is essential for protein export out of the ER (Novick et al 1981, Kaiser & Schekman 1990, Barlowe et al 1993, Kuge et al 1994).

Tagging of COPII components with GFP has enabled direct visualization of the dynamics of ER exit sites in living cells. When expressed in mammalian cells, Sec13-GFP and Sec24D-GFP localize to sites where VSVG first concentrates upon release from the ER, indicating that these GFP chimerae label functional ER exit sites (Roberts et al 1999, Stephens et al 2000). The ER exit sites visualized with COPII-GFP chimerae are found to label hundreds of small structures distributed stochastically along the ER (Hammond & Glick 2000, Stephens et al 2000). Time-lapse imaging has revealed that ER exit sites exhibit very little movement and are long-lived, consistent with their acting as fixed domains where secretory cargo is recruited and concentrated. Newly synthesized secretory cargo

molecules accumulate at COPII-labeled sites and then move away from them. Under conditions of synchronized release of cargo (i.e. VSVG), ER exit sites do not exhibit long-range movements toward the Golgi region, nor does their overall distribution and number appear to be affected by this cargo movement. This indicates that ER exit sites are not consumed by the formation and translocation of pre-Golgi transport intermediates and that changes in cargo flux out of the ER are easily accommodated by these structures.

Photobleaching of Sec24D-GFP-labeled ER exit sites has shown that the bleached chimerae at these sites exchange with nonbleached molecules in the cytosol very quickly (<1 min) at 37°C (Stephens et al 2000). Repetitive association/dissociation with the same region of membrane would explain the longevity of ER exit sites as distinct domains on the ER. Whereas ER exit sites are more or less stationary and randomly localized under steady state, they can change their distribution upon disruption of Golgi-to-ER recycling pathways. For example, depolymerization of microtubules causes Sec13-labeled exit sites to cluster next to Golgi elements, suggesting that their organization and assembly is coupled in some way to retrograde membrane traffic from the Golgi complex (Hammond & Glick 2000). These observations imply that despite the temporal stability of ER exit sites, they are dynamic structures that can rapidly reorganize in response to trafficking perturbants.

Further insight into the dynamics of the molecular machinery associated with ER exit sites has come from studies visualizing GFP-tagged soluble NSF attachment protein receptors (SNAREs), including rsec22b and rbet1 (Chao et al 1999), that localize at these sites. Because binding of SNARES on opposite membranes is thought to bring the bilayers in close proximity so that fusion can occur (Söllner et al 1993, Nichols et al 1997, Hanson et al 1997, Lin & Scheller 1997), the localization of rsec22b and rbet1 to ER exit sites suggests that they have a role in fusion events involved in the formation of nearby pre-Golgi intermediates. Fluorescence from the chimerae appears to reside primarily on membranes that closely resemble ER exit sites because the membranes do not generally translocate with cargo into the Golgi region and remain at relatively stationary sites, akin to the dynamics of GFP-tagged Sec13 membranes (Chao et al 1999). One explanation for this observation is that the chimerae are locally retrieved and recycled back to the ER so that they can be continually reutilized in the membrane fusion events at ER exit sites required for the formation of pre-Golgi transport intermediates.

Pre-Golgi Intermediates

When secretory cargo is concentrated at ER exit sites through the activity of COPII and other components, these sites are believed either to engage in repeated vesicle budding and delivery to pre-Golgi intermediates (Bannykh et al 1996) or to directly transform into tubular membrane clusters (Clermont et al 1994, Krijnse-Locker et al 1994). Originally, pre-Golgi intermediates were thought to be a stable compartment from which small vesicles bud for delivery of secretory

cargo to the Golgi complex (Lotti et al 1992). Pre-Golgi intermediates, however, are now recognized as transport vehicles for protein delivery to the Golgi complex. This was demonstrated in studies where transport of VSVG-GFP through the secretory pathway was visualized in living cells (Presley et al 1997, Scales et al 1997). When VSVG-GFP expressing cells are shifted from 40 to 32°C, to allow correct folding and assembly of VSVG, the chimerae become concentrated within minutes into bright, fluorescent pre-Golgi structures localized adjacent to ER exit sites. The structures then translocate through the cytoplasm with no loss of fluorescence intensity. During movement toward the Golgi complex from the ER they often show deformation of shape, including the extension of long tubular processes, which indicates that these intermediates are pleiomorphic and not single vesicles. The pre-Golgi structures are associated with microtubules and move in a minus-end-directed manner mediated by dynein. The fact that pre-Golgi structures move as discrete entities away from ER exit sites suggests that intermediates are constantly being generated de novo at these sites rather than existing there as a stable intermediary compartment. Furthermore, because pre-Golgi structures seem to fuse with the Golgi complex following transport, they appear to have only a transient existence.

Pre-Golgi intermediates sort and recycle selected components back to the ER (Tang et al 1995, Klumperman et al 1998). This can now be visualized in vivo by following the dynamics of GFP-tagged p58 (Roberts et al 1999), the rat homologue of ERGIC53 (Lahtinen et al 1992, Hauri et al 2000), which is a lectin-like protein that constitutively cycles between the ER and the Golgi, and is thought to have a role in forward transport of specific cargo out of the ER (Nichols et al 1998, Vollenweider et al 1998, Appenzeller et al 1999, Moussalli et al 1999). p58-GFP is found to co-localize with VSVG in pre-Golgi intermediates when ER to Golgi trafficking is inhibited at 15°C; upon warming p58-GFP rapidly moves out of these structures into long tubule processes that extend outward rather than in toward the Golgi complex (Roberts et al 1999). The tubule processes soon disappear in the cell periphery, most likely as a result of fusion with the ER. The fact that p58-GFP fluorescence does not move out of pre-Golgi intermediates as punctate spots, but rather as tubules, suggests that the recycling intermediates carrying p58-GFP also have a tubular rather than vesicular nature.

COPI Functions and Dynamics

COPI is a heptameric cytosolic protein complex that, in conjunction with the ras-like GTP-binding protein ARF1, assembles onto Golgi membranes to form an electron-dense coat thought to facilitate membrane budding and fission of transport intermediates (Donaldson et al 1990, Serafini et al 1991, Pepperkok et al 1993). Originally, COPI binding was proposed to mediate the formation of transport vesicles carrying anterograde cargo from either pre-Golgi intermediates or Golgi cisternae (Rothman 1994). However, no tiny vesicles that are enriched in GFP-tagged cargo molecules (which would appear as punctate fluorescence for

spherical vesicles 50–100 nm in diameter) are seen budding off pre-Golgi intermediates or tracking into the Golgi complex in imaging studies (Scales et al 1997, Presley et al 1997). Moreover, aggregates of cargo have been shown in electron microscopy studies to progress with cisternae through Golgi stacks without using vesicular intermediates (Melkonian et al 1991, Clermont et al 1993, Bonfanti et al 1998). These observations, coupled with the fact that some COPI mutant alleles cause secretion of ER/Golgi resident proteins rather than a block in forward traffic (reviewed in Pelham 1994, Gaynor et al 1998), have raised doubts over the original proposed function of COPI in the budding of anterograde vesicles.

An alternative role suggested for COPI is for mediating the budding of retrograde vesicles, which would recycle proteins back to the ER or from late to early Golgi compartments (Pelham 1994). The dual findings that COPI subunits can bind dilysine motifs (Cosson & Letourneur 1994), which are believed to function as ER retrieval sequences on proteins (Letourneur et al 1994), and that mutant COPI subunits that do not bind these motifs result in delivery of dilysine-tagged reporters to the cell surface (Letourneur et al 1994) have provided some evidence for believing that COPI mediates retrograde traffic. However, these findings show only a correspondence of COPI-binding to retrieval of dilysine-containing proteins. Dilysine motifs are present on many proteins that never leave the ER, as well as on Golgi-resident proteins that engage in bidirectional transport between the ER and Golgi complex (Teasdale & Jackson 1996, Sohn et al 1996, Dominguez et al 1998, Nickel et al 1998). What specifies their interaction with COPI in different membrane compartments and whether such interactions result in their retention or retrieval are unclear. It thus remains to be established whether membrane-bound COPI gathers cargo into coated vesicles that are retrograde-directed, forward-directed, or both.

An additional function of COPI binding might be, therefore, to laterally segregate proteins and lipids into separate domains that subsequently bud off as forward or retrograde transport intermediates. This possible role would explain why, when coat assembly is blocked with ARF1 inhibitory mutants or by BFA treatment (Donaldson et al 1992, Helms & Rothman 1992, Dascher & Balch 1994), membrane traffic is not inhibited per se but is deregulated, resulting in Golgi proteins nonselectively entering a tubule pathway back to the ER (Lippincott-Schwartz et al 1998). It is also consistent with the finding that injection of anti-COPI antibodies does not block recycling of all proteins back to the ER but only a subset (Girod et al 1999).

The use of GFP fusion proteins in living cells is helping to clarify these proposed functions of COPI. By following the progress of VSVG-GFP as it exits the ER, it was found that while the chimerae initially concentrates in COPII-positive structures, these structures mature to pre-Golgi intermediates through a process involving replacement of COPII with COPI (Scales et al 1997, Stephens et al 2000). This would suggest a sequential mode of action of the coats (Rowe et al 1996, Scales et al 1997, Stephens et al 2000); whereby COPI association occurs after membrane sites have been first differentiated by COPII.

Evidence that COPI association helps to further mature COPII-derived ER exit sites has come from a cell-free assay reconstituting ER export. Lavoie et al (1999) found that COPI binding to dilysine-containing p24 family members in ER membranes is essential for cargo exit site formation. A functional role for COPI in the biogenesis of ER exit sites would explain why, when COPI is prevented from binding to membranes [e.g. in cells expressing ARF dominant-negative mutants or in cells treated with BFA, which inhibits ARF activation (Donaldson et al 1992, Dascher & Balch 1994)], cargo proteins such as VSVG, as well as ER-localized Golgi enzymes, fail to efficiently concentrate at ER exit sites even though Sar1/COPII still binds and ERGIC53 can concentrate (Lippincott-Schwartz et al 1998). In the absence of COPI activity, therefore, the action of Sar1/COPII is insufficient to sort into ER exit sites, and thereby into pre-Golgi intermediates, the diverse array of molecules required to build and maintain these structures. The fact that, in the absence of COPI binding to membranes, many proteins within pre-Golgi and Golgi membranes redistribute back into the ER via retrograde tubule carriers (Lippincott-Schwartz et al 1990, Sciaky et al 1997) further suggests that COPI binding is required for maintaining these molecules in pre-Golgi and Golgi elements after they have formed, for example, by suppressing tubule formation.

Imaging of cells expressing εCOP-tagged with GFP has shown that a large amount of COPI associates with pre-Golgi structures in addition to Golgi membranes and that COPI remains localized to pre-Golgi structures as they track into the Golgi region (Presley et al 1998, Shima et al 1999). Because this chimera is able to rescue the temperature-sensitivity of ldlF cells, which are defective for εCOP, it is assumed that εCOP-GFP mimics the behavior of wild-type εCOP. Dual-color imaging and immuno-electron microscopy studies have further shown that within pre-Golgi intermediates, COPI is segregated from anterograde cargo but not from KDEL receptor (which recycles back to the ER) (Shima et al 1999, Martínez-Menárguez et al 1999). Retrograde tubule processes containing KDEL receptor or p58 that extend from pre-Golgi or Golgi structures are relatively depleted of COPI fluorescence (Shima et al 1999, Roberts et al 1999), and there are no apparent COPI-containing small spherical vesicles moving out from these structures (Shima et al 1999).

Photobleaching of εCOP-GFP-labeled pre-Golgi or Golgi structures revealed that there is rapid exchange between cytosolic and membrane-bound pools of COPI complexes (Presley et al 1998). This is analogous to results seen with ARF1-GFP (Vasudevan et al 1998). The rapid equilibrium of ARF1-GFP between cytoplasm and membrane is dependent on ARF GTPase activity, and the rate-limiting step for membrane dissociation of ARF-GFP is hydrolysis of bound GTP. Cycling of COPI-GFP on and off membranes occurs every 30 s and is also dependent on the ARF guanine nucleotide-binding state. Interestingly, the cycling rate of COPI is only slightly diminished at very low temperatures (i.e. 4°C) where vesicle transport does not occur (Presley et al 1998). This indicates that COPI association/dissociation with membranes is not directly coupled to vesicle budding

and release from membranes and is likely to have roles other than simply vesicle formation. As suggested above, one possible role is in differentiating ER exit sites and in generating and maintaining pre-Golgi and Golgi elements by lateral recruitment and retention of specific proteins and lipid into discrete membrane domains.

More work needs to be done to fully clarify the role(s) of COPI in secretory transport and organelle maintenance and biogenesis. Use of GFP chimerae in live-cell imaging is likely to facilitate this endeavor by allowing the temporal and spatial dynamics of COPI, as well as other regulatory machinery, to be dissected. For example, studies of the in vivo dynamics of GFP-tagged p24A and p23, which are thought to act as integral receptors for the COPI coat through cytosolic dilysine motifs (Stamnes et al 1995, Sohn et al 1996, Dominguez et al 1998), show that these proteins constitutively cycle between the ER and Golgi and move within pleiomorphic transport intermediates (Blum et al 1999). Whether overexpressing these proteins or retaining them in the ER affects the in vivo dynamics and/or distribution of COPI, for example, will be important to address in future work aimed at understanding the relationship between p24 family members and COPI.

DYNAMICS OF THE GOLGI COMPLEX

The Golgi complex occupies a central position in the secretory pathway. It is the site where proteins and lipids are modified and sorted, and it acts as a filter to segregate proteins and lipids to be retained in the ER/Golgi system from those to be delivered to the plasma membrane. Transport intermediates carrying cargo derived from the ER deliver their contents uniquely to the *cis* face of the Golgi complex, which exists as an elaborate tubular network. The cargo molecules then move through polarized stacks of flattened cisternae enriched in glycoprotein and glycolipid-processing enzymes to the TGN where they are packaged into membrane-bound carriers destined for the plasma membrane. The forward flow of secretory cargo through the Golgi is balanced by backward flow of selected components to the ER.

The findings that pre-Golgi intermediates undergo maturation (by recycling of selected components back to the ER) (Tang et al 1995, Klumperman et al 1998), are capable of homotypic fusion (Presley et al 1997), and track into the Golgi region (Presley et al 1997, Scales et al 1997) has led to the idea that they are the direct precursors of Golgi elements, with Golgi cisternae formed by continuous maturation and differentiation of pre-Golgi intermediates (Bannykh & Balch 1997, Glick et al 1997, Mironov et al 1997, Pelham 1998). Under this view, the *cis* face of the Golgi would be the site where pre-Golgi intermediates first merge together, and the *trans* face would represent older cisternae in the process of segregating and packaging protein and lipid into post-Golgi transport intermediates. Recycling pathways would ensure that resident Golgi enzymes are retained within this system. This view of the Golgi complex would explain the existence of polarized Golgi entry and exit faces and why the overall distribution of the Golgi varies considerably

between cell types. In many plants and fungi, for example, Golgi stacks are scattered throughout the cell, whereas in mammalian fibroblasts, which translocate pre-Golgi intermediates from the periphery, Golgi stacks are clustered together in a juxtanuclear array at the minus ends of microtubules. Studies using GFP chimerae to analyze the dynamics of protein movement associated with the Golgi complex are providing important support to this view of the Golgi complex and are giving insight into the mechanisms underlying Golgi maintenance and dynamics throughout the cell cycle.

Golgi Morphology and Behavior in Different Cell Types

The behavior of Golgi elements in plant cells has been visualized with GFP-tagged Golgi resident proteins (Boevink et al 1998, Nebenführ et al 1999). Golgi stacks move rapidly and extensively along the ER network throughout the cytoplasm, never departing from ER tubules. The ER tubules are aligned along the actin cytoskeleton, precisely matching its architecture. Treatment of cells with agents that disrupt the actin network inhibits Golgi movement but does not dissociate Golgi elements from the ER. This suggests that movement of Golgi units along the ER occurs by an actin-based motility mechanism. The intimate positioning of ER and Golgi membranes in plant cells is presumably critical for efficient delivery of cargo molecules to the Golgi and retrieval of selected protein and lipid to the ER because plant cells lack radially arranged microtubules for moving transport intermediates through the cytoplasm.

Time-lapse studies of Golgi membranes in the yeast strain *Saccharomyces cerevisiae* have revealed that, as in plant cells, Golgi membranes consist of scattered, moving elements (Wooding & Pelham 1998). Whether the yeast Golgi elements move along the surface of the ER is not known. However, in a different yeast strain, *Pichia pastoris*, Golgi stacks containing both early and late Golgi markers are stably localized next to ER exit sites (Rossanese et al 1999). Interestingly, early and late Golgi markers in *S. cerevisiae* are found in distinct structures, raising the possibility that they represent scattered Golgi cisternae at different stages of maturation. To gain insight into the transport processes associated with these structures, Wooding & Pelham (1998) looked at changes in Golgi markers in response to mutations that affect secretory traffic. Both early and late Golgi markers disperse into vesicular structures within minutes of temperature shift in *sec18*, *sft1*, and *sed5* conditional mutants (where membrane fusion and sorting events are inhibited), but not in *sec14* cells (where transport from the Golgi to the plasma membrane is blocked). They have also found that the early Golgi marker, Sed5p, redistributes into the ER in *sec12* cells (where ER export is blocked). These results emphasize the dynamic nature of the Golgi complex and provide support for the concept that resident Golgi components undergo continuous recycling either within the Golgi complex or back to the ER, as anterograde cargo advances.

In mammalian cells, time-lapse imaging of the Golgi complex with GFP-tagged Golgi enzymes has revealed a juxtanuclear arrangement of Golgi elements that is

relatively stable over long time periods (Sciaky et al 1997). Nevertheless, local remodeling of Golgi stacks is occurring constantly and involves the formation and/or detachment of thin tubule processes that interconnect adjacent Golgi elements or move out from the Golgi along microtubules (Sciaky et al 1997, Polishchuk et al 1999). Tubules that break off from the Golgi move at rates of 0.6 μm/s along microtubules to the cell periphery (Sciaky et al 1997). In the cell periphery, detached Golgi tubules are often seen curling up and then disappearing from view (Sciaky et al 1997), which suggests they have fused with the ER, since a small population of Golgi enzymes resides in the ER (Zaal et al 1999). Microtubule depolymerization inhibited the appearance of tubule connections between Golgi stacks and the extension of peripheral tubules (Sciaky et al 1997, Polishchuk et al 1999). These results suggest that Golgi membrane tubules are a prominent feature of Golgi dynamics and play dual roles in interconnecting adjacent Golgi stacks and in retrograde traffic back to the ER.

Diffusional Mobility of Golgi Resident Proteins

FRAP studies using GFP-tagged Golgi resident enzymes have shown that these proteins are highly mobile in Golgi membranes with no constraints to their lateral diffusion (e.g. D ranging between 0.3 and 0.5 $\mu m^2/s$ and 90% of the molecules mobile) (Cole et al 1996b), implying that these molecules are not normally trapped in aggregates. Fluorescence recovery in these experiments is from lateral diffusion of the chimerae in the Golgi bilayer and not from intra-Golgi vesicle trafficking because no change in D or mobile fraction is observed under conditions where vesicle transport is impeded, including ATP depletion and reduced temperatures. Conditions that cause extensive and irreversible binding of peripheral protein complexes like COPI to Golgi membranes, including treatment with AlF and GTPγS, immobilize the chimerae. Golgi stacks in many cells are extensively interconnected, since repetitive photobleaching of a small area eliminates all Golgi fluorescence over time (Cole et al 1996b). In cells whose Golgi elements are fragmented and not connected by tubular elements, however, repetitive bleaching leaves pockets of fluorescence, even between elements that appear in close proximity. These findings suggest that there is significant lateral diffusion rather than vesicle trafficking of Golgi enzymes between Golgi stacks in mammalian cells (Cole et al 1996b). The transient or permanent connections of Golgi cisternae by membrane tubules observed in time-lapse imaging (Sciaky et al 1997) and electron microscope studies (Rambourg & Clermont 1990, Clermont et al 1994) are likely to mediate the extensive lateral exchange of resident proteins between Golgi stacks.

An important issue raised by these studies is the degree of connectivity between Golgi stacks: Do they only interconnect homologous Golgi cisternae *cis* to *cis* and *trans* to *trans*? If they do not, and there is a high degree of connectivity, then extensive mixing of Golgi membrane components should result in an interconnected Golgi system. How could secretory cargo pass through such a system? One possibility is that the polarized entry and efflux domains of Golgi stacks

give rise to a directional membrane flow, with cargo diffusing across this system via the extensive tubule connections between stacks (Mironov et al 1997, Sciaky et al 1997). Golgi enzymes could diffusively self-organize within this system since substrates for early acting enzymes are available upon entry into the Golgi, and substrates for later-acting enzymes arise when cargo has diffused deeper into Golgi stacks. In this way forward-moving cargo molecules could help spatially organize mobile Golgi enzymes into compartmentalized multi-protein complexes (Jungmann & Munro 1998) as cargo is sequentially processed by these enzymes. The profound differences between this view of Golgi organization and trafficking and other more traditional models, which assume that the generation and maintenance of functionally discrete Golgi cisternae or subcompartments requires vesicle budding and fusion (Rothman & Wieland 1996, Glick et al 1997), underscore the need for further investigation in this area.

Golgi Remodeling Through Protein Cycling Pathways to and from the ER

Virtually all conditions that interfere with delivery of proteins from the ER to the Golgi complex, including treatments with BFA, okadaic acid, and overexpression of particular rab, Sar1, and ARF mutants, result in dispersal of Golgi membranes (Lippincott-Schwartz et al 1990, Lucocq 1992, Martinez et al 1997, Storrie et al 1998). Moreover, microtubule disruption, which prevents peripheral pre-Golgi intermediates from tracking into the Golgi region, causes Golgi proteins to reversibly redistribute to numerous peripheral sites. These observations highlight the Golgi's dynamic nature and imply that a constant influx of membrane from the ER is required to maintain Golgi structure. Because Golgi proteins often redistribute to the site where membrane traffic is inhibited during these perturbations, the data have raised the possibility that Golgi proteins, which are highly mobile in Golgi cisternae, undergo constitutive cycling to and from the ER (Cole et al 1996a, Storrie et al 1998, Zaal et al 1999). Imaging studies with GFP chimerae have helped distinguish between this possibility and an alternative view (Shima et al 1998) that redistribution of Golgi components under these conditions occurs independently of effects on ER/Golgi membrane trafficking pathways.

To investigate the mechanism underlying Golgi fragmentation in cells whose microtubules are disrupted, Storrie et al (1998) performed time-lapse imaging of GFP-tagged Golgi markers in cells treated with nocodazole to visualize the fragmentation process. If fragmentation arises by direct breakdown of the Golgi complex (e.g. by Golgi stacks no longer being tethered together by microtubules), then small fragments would be expected to disperse out from the Golgi region once microtubules are disrupted. This was not observed. Instead, scattered but stationary Golgi elements appear abruptly with no Golgi fragments seen tracking outward from the compact, juxtanuclear Golgi complex. The scattered Golgi stacks arise next to ER exit sites that can be loaded with secretory cargo released from the ER (Cole et al 1996a), as expected if they are formed by cycling of Golgi enzymes

back to the ER and re-emergence at ER exit sites. Importantly, this was confirmed when microinjection of the dominant-negative mutant of Sar1, which blocks ER export, prevented the Golgi stacks from forming (Storrie et al 1998). Further evidence that Golgi fragments in nocodazole-treated cells are derived from the ER rather than from pre-existing Golgi stacks was obtained by Zaal et al (1999). They selectively photobleached Golgi fluorescence in cells expressing a GFP-tagged Golgi enzyme, galactosyltransferase (GalTase-GFP) and still observed numerous bright peripheral fragments enriched in GalTase-GFP in cells treated with nocodazole. Since Golgi fluorescence was photobleached at the time nocodazole was added to cells, accumulation of GalTase-GFP in peripheral Golgi structures comes only from the ER pool of GalTase-GFP (which was not photobleached) and not from direct fragmentation or dispersal of Golgi membranes.

The above results support the idea that Golgi protein cycling pathways involving the ER as an intermediate underlie the fragmented Golgi phenotype in nocodazole-treated cells. In the absence of microtubules, Golgi proteins that have recycled back to the ER are exported into pre-Golgi intermediates that subsequently fail to assemble in the perinuclear Golgi complex. Maturation of these de novo structures into small Golgi stacks enables the cell to re-establish secretory flow from the ER into the Golgi complex when microtubules are absent.

To provide evidence that Golgi proteins cycle through the ER constitutively in the absence of cellular perturbants, Zaal et al (1999) selectively photobleached Golgi or ER pools of GalTase-GFP and monitored recovery from the nonbleached pool in the absence of protein synthesis. Within 20 min of selectively photobleaching the ER pool of GalTase-GFP, ER membranes were found to regain much the same percentage of total cellular fluorescence observed before photobleaching. A similar recovery was found when the Golgi pool of GalTase-GFP was selectively photobleached. This indicated that the steady-state concentrations of GalTase-GFP in Golgi and ER membranes arise by continuous cycling rather than by GalTase-GFP residing stably within these compartments. Kinetic modeling of the FRAP experiments revealed that an average GalTase-GFP molecule cycles between Golgi and ER every 85 min, residing in the Golgi for approximately 58 min and in the ER for 27 min. Evidence that other Golgi enzymes also constitutively cycle between ER and Golgi has come from experiments in cells expressing mutant Sar1, which blocks ER export (Storrie et al 1998, Zaal et al 1999). These studies showed that the majority of Golgi enzymes become trapped in the ER within 3 to 5 h of the block, suggesting most Golgi enzymes recycle back to the ER within this time frame.

Is Golgi Protein Recycling to the ER Mediated by Specific Signals?

Many proteins known to cycle between the ER and Golgi contain dibasic signals within their cytoplasmic domain, which are thought to be recognized by a sorting machinery that mediates retrieval from the Golgi complex (Letourneur et al 1994).

To test whether specific signals on Golgi proteins are required for them to recycle, Cole et al (1998) developed a retrograde transport assay based on the capacity of the ER to retain misfolded proteins. The lumenal domain of ts045 VSVG was fused to Golgi or plasma membrane targeting domains without disrupting the thermoreversible folding character of VSVG or the ability of the modified proteins to target and function properly. Upon shift to nonpermissive temperature in this assay, proteins able to recycle become trapped in the ER as they cycle through it; proteins that are stably retained in the Golgi or on the plasma membrane can not. This occurs because of the unique capacity of the ER to retain misfolded VSVG. It was found that Golgi-localized fusion proteins, but not VSVG itself, redistribute back to the ER upon shift to nonpermissive temperature, where they become misfolded and are retained (Cole et al 1998). This occurs between 15 min and 2 h after temperature shift, depending on the chimera, and does not require new protein synthesis. Recycling of the chimerae does not require specific signals but is dependent on the length of the transmembrane domain of the construct: When the 23 amino acid transmembrane domain of VSVG is replaced with a synthetic domain of 15 leucines, the resulting construct is found to undergo retrograde transport.

Many Golgi resident proteins have shorter transmembrane domains than molecules targeted to the plasma membrane (Bretscher & Munro 1993). This has led to a lipid-sorting model for Golgi protein localization where Golgi proteins are proposed to partition into thinner regions of the Golgi bilayer and are excluded from thicker regions that are enriched in sphingolipid and sterols destined for the plasma membrane (Bretscher & Munro 1993, Munro 1995). The finding that shortening the transmembrane domain of VSVG leads to its localization within the Golgi and constitutive recycling to the ER (Cole et al 1998) suggests that lipid partitioning also underlies entry of proteins into the Golgi-to-ER retrograde transport pathway. Under this view, specific retrieval determinants (e.g. KDEL and KKXX) could increase the efficiency of recycling of specific proteins, but the overall extent of retrograde traffic would operate independently of these types of signals.

What Type of Structures Mediate Golgi to ER Retrograde Transport?

Imaging of GFP-tagged proteins that cycle between the Golgi and ER suggests that retrograde transport intermediates are characteristically tubular. In time-lapse experiments, membrane tubules carrying KDELR-GFP, p58-GFP, GalTase-GFP or Cy3-shiga toxin B fragment (StB) were found to extend out from the Golgi, break off, and then move peripherally at rates of 0.6 to 1.0 μm/s along microtubules (Sciaky et al 1997, Roberts et al 1999, White et al 1999). KDELR-GFP and p58-GFP were observed in peripheral tubules more frequently than GalTase-GFP, consistent with their recycling to the ER at a higher rate (Sciaky et al 1997, Roberts et al 1999). Not all retrograde tubules are alike, however. In double-label experiments,

retrograde tubules carrying StB do not contain KDELR-GFP but are enriched in Rab6, a ras-like GTPase that has been proposed to function in Golgi-to-ER trafficking (White et al 1999). According to White et al this implicates two separate retrograde carriers for delivery of proteins back to the ER. Retrograde tubules appear comparatively simple when contrasted with anterograde pre-Golgi intermediates, which are enriched in COPI and mature by recycling specific components as they translocate inward to the Golgi complex.

Retrograde tubules extending out from the Golgi complex proliferate in cells treated with BFA (Sciaky et al 1997), which inhibits nucleotide exchange onto ARF (Peyroche et al 1999) and leads to loss of ARF1 and COPI binding to membranes (Donaldson et al 1991). The tubules are longer, contain a mixed population of Golgi protein and lipid, and do not detach as readily from the Golgi complex (Sciaky et al 1997). Ultimately, one or more of the tubules fuses with the ER into which the Golgi is then absorbed. The unidirectionality of Golgi transfer into the ER, its speed (between 15 and 30 s), and its irreversibility in BFA-treated cells suggest that the ER provides a lower-energy environment for membrane protein and lipid than does the Golgi system (Sciaky et al 1997). The specific chemical basis for such an energy difference between ER and Golgi membranes is unknown but could arise from the activities of the Sar1/COPII and ARF/COPI sorting machinery, which help to concentrate and segregate membrane proteins exported from the ER in an energy-dependent manner (Rowe et al 1996, Kuehn & Schekman 1997), thereby increasing the potential energy of Golgi-destined membranes.

Golgi Breakdown and Reassembly in Mitosis

In mammalian cells, the Golgi breaks down and then reassembles during mitosis. One proposal is that this depends on a process of continual budding and inhibition of fusion of Golgi transport vesicles (Warren 1993). At the end of mitosis, Golgi vesicles and fragments are proposed to fuse together after stochastically partitioning into daughter cells (Lucocq & Warren 1987, Shima et al 1998). Recent fluorescence imaging studies using GFP chimerae to visualize Golgi disassembly and reassembly have led to an alternative model in which the Golgi is absorbed into and then re-emerges from the ER during mitosis (Zaal et al 1999).

Time-lapse imaging of cells expressing GalTase-GFP as a Golgi reporter revealed that Golgi breakdown and reassembly during mitosis occur through a series of four discrete stages (Zaal et al 1999): (*a*) the loss of a centralized Golgi ribbon and appearance of peripheral Golgi fragments at prophase, (*b*) loss of Golgi fragments and the appearance of Golgi proteins in widely dispersed membranes between metaphase and telophase, (*c*) reappearance of small peripheral Golgi fragments during late telophase to early cytokinesis, and (*d*) coalescence of Golgi fragments into juxtanuclear sites at cytokinesis (see Figure 3). An earlier time-lapse imaging study of mitotic Golgi breakdown and reassembly using GFP chimerae found similar progressive stages but did not report the phenotype associated with stage (*b*), where Golgi proteins are widely dispersed within the cytoplasm (Shima

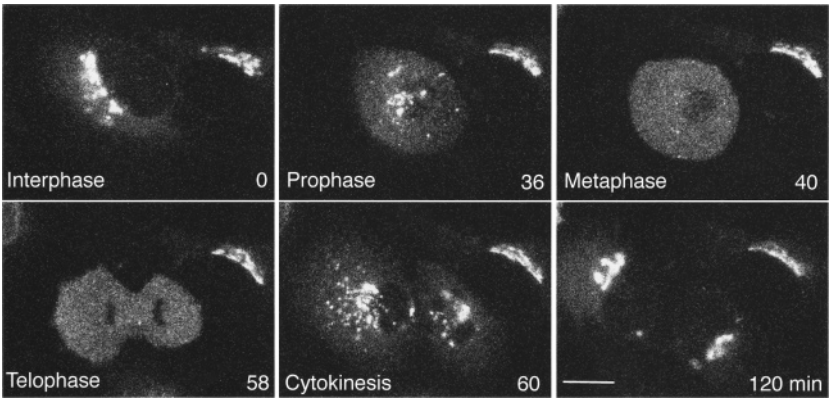

Figure 3 Mitotic Golgi breakdown and reassembly visualized in HeLa cells expressing GalTase-GFP. Bar = 10 μm.

et al 1998). One explanation for the discrepancy in results concerning stage (*b*) is that the fluorescence associated with mitotic Golgi fragments was not quantitated in the earlier study, and thus the percentage of total GFP-fluorescence residing in these fragments relative to dispersed fluorescence could not be compared. Zaal et al found that when Golgi fragments persisted in metaphase cells, the percentage of GFP chimera fluorescence residing in these structures is less than 10% of total fluorescence even when the fragments appeared bright. This finding, plus the fact that most endogenous Golgi proteins detected by immunolabeling show a dispersed phenotype in metaphase/anaphase (Jesch & Linstedt 1998, Hammond & Glick 2000), suggests that Golgi proteins at this stage of mitosis are, in general, in membranes widely dispersed throughout the cell.

To determine the nature of these dispersed membranes, Zaal et al (1999) found that immuno-EM labeling of cells specifically selected at this stage of mitosis showed clear ER staining both for GFP-Golgi chimerae as well as for native Golgi proteins. Photobleaching experiments using Golgi-GFP fusion proteins in living cells ruled out the possibility that, in addition to ER, a population of Golgi proteins at this stage resides in vesicles. Because ER export stops in mitosis (Featherstone et al 1985), one explanation for the ER localization of Golgi proteins in mitosis is that it represents Golgi proteins that have cycled back to the ER and become trapped. Consistent with this possibility, when ER export cannot resume at the end of mitosis through mutant Sar1 expression, Golgi structures do not reform (Zaal et al 1999). These results suggest that a reversible alteration of constitutive cycling pathways connecting the ER and Golgi underlies Golgi dispersal and reformation during mitosis. Thus, when ER export stops upon entry into mitosis, Golgi structure is lost because recycling Golgi proteins become trapped in the ER, and Golgi structure is regained only when ER egress resumes at the end of mitosis. Current biochemical work defining the machinery and signaling pathways leading

to mitotic Golgi disassembly and reassembly (Acharya et al 1998, Lowe et al 1998, Farmaki et al 1999) should help clarify the molecular basis for alterations in ER/Golgi cycling during mitosis.

GOLGI TO PLASMA MEMBRANE TRANSPORT

Upon reaching the *trans* Golgi network of the Golgi complex, protein and lipid are packaged into transport intermediates that move through the cytoplasm to fuse with the cell surface or with other compartments of the endosomal system. Export of anterograde cargo out of the TGN is not a bulk flow process but involves sorting into distinct pathways (Keller & Simons 1997). The mechanisms and carriers involved in exocytic transport are being clarified using GFP chimerae. Such work has revealed that post-Golgi carriers (PGCs) are large, pleiomorphic tubular structures in addition to small vesicles. Moreover, they fuse with the plasma membrane without intersecting other membrane pathways in the cell. A wide variety of cargo molecules have been identified in such structures, including apical- and basolateral- targeted plasma membrane proteins, soluble cargo, synaptic vesicle proteins, and lipid-anchored proteins (Wacker et al 1997, Nakata et al 1998, Hirschberg et al 1998, Toomre et al 1999, Choy et al 1999, Polishchuk et al 2000, Kreitzer et al 2000).

Formation of Post-Golgi Carriers

How do PGCs arise from the Golgi? High-resolution confocal imaging following shift to permissive temperature has shown that VSVG-GFP initially segregates into discrete domains on Golgi membranes (Hirschberg et al 1998, Toomre et al 1999). These domains then elongate into tubules before detaching from the Golgi body to form a PGC (Hirschberg et al 1998) (see Figure 4). Because TGN resident proteins such as furin are excluded from the VSVG-GFP-enriched tubules when they are still attached to Golgi membranes (Hirschberg et al 1998), anterograde cargo appears to be actively sorted into tubular domains during export out of the Golgi.

Do different cargo molecules sort into different PGCs? Dual-color time-lapse imaging with cyan and yellow spectral variants of GFP has been used to address this question (Keller et al 1998). These studies show that basolateral cargo (VSVG) laterally segregates from apical cargo (GPI) on the surface of Golgi elements to give rise to distinct PGCs enriched in either VSVG or GPI, which then translocate outward before fusing with the plasma membrane. The observed sorting of basolateral and apical cargo proteins prior to export from the Golgi raises important mechanistic questions: Does sorting require signal recognition? Is partitioning of proteins into preferential TGN lipid domains or rafts sufficient? Because the segregation of cargo into domains and formation of distinct PGCs occur as rapid, dynamic processes, dual-color time-lapse imaging of GFP chimerae is likely to

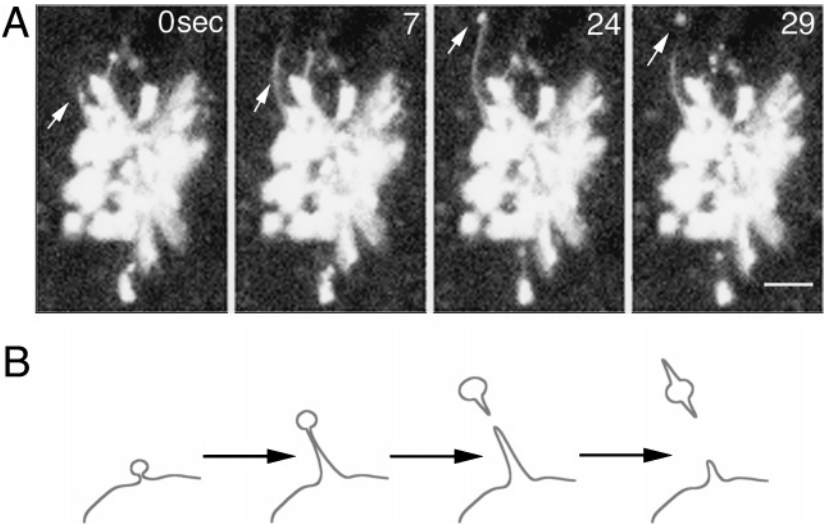

Figure 4 The formation and budding of post-Golgi carriers. Confocal images of a cell expressing VSVG-GFP approximately 50 min after shift from 40 to 32°C. Arrows point to a tubular membrane pulling off and detaching from the Golgi cisternae. The scheme illustrates the formation and the budding of a PGC.

be an important tool in understanding how these processes relate to sorting of basolateral and apical cargo.

After extending out from Golgi membranes, post-Golgi cargo-containing tubules are severed. Dynamin-2, a TGN-localized GTPase and mechanoenzyme (Jones et al 1998), appears to play a role in this severing process. In cells co-transfected to express a dominant-negative dynamin-2 with GFP-tagged cargo, it was found that delivery of cargo to the plasma membrane is impeded and that cargo accumulates in anastomosing tubular arrays extending out from the Golgi complex (Kreitzer et al 2000, Cao et al 2000). In addition to dynamin-2, an actin-based cytoskeletal system seems to participate in severing of the Golgi-derived tubules involved in plasma membrane transport (Müsch et al 1997) because cytochalasin B treatment (which disrupts the actin cytoskeleton) slows down traffic from Golgi to the plasma membrane and causes Golgi-derived tubules containing VSVG-GFP to become longer and detach more slowly from the Golgi (Hirschberg et al 1998).

Dynamics of PGCs

After detaching as tubules from the Golgi complex, PGCs undergo dramatic shape changes, including extension, retraction, and bifurcation (Nakata et al 1998, Hirschberg et al 1998, Toomre et al 1999, Polishchuk et al 2000). In COS cells, PGCs often extend as 3–5 μm-long tubules into the cell periphery, have an average surface area of 1.3 μm^2, and carry up to 10,000 VSVG-GFP molecules (Hirschberg

et al 1998). A 100-nm vesicle of equal surface density, by contrast, would carry only about 100 VSVG-GFP molecules. Correlative light-electron microscopy, a technique in which structures observed in vivo are then fixed and examined by electron microscopy, has revealed that PGCs can be as large as half the size of Golgi cisternae and that they exist as tubular-saccular structures (Nakata et al 1998, Polishchuk et al 2000). It is clear that cargo molecules like VSVG-GFP are carried to the plasma membrane primarily in such large tubular structures rather than in small vesicles because quantitative fluorescence imaging experiments show that the majority of total VSVG-GFP fluorescence is delivered to the plasma membrane in such structures (Hirschberg et al 1998, Toomre et al 1999). Peripheral movement of PGCs occurs at rates up to 2.7 μm/s and requires microtubules (Nakata et al 1998, Hirschberg et al 1998, Toomre et al 1999) and the activity of the plus-end directed motor protein kinesin (Kreitzer et al 2000). Moreover, the lifetime of PGCs is only 3.8 min on average in COS cells (Hirschberg et al 1998), and they do not fuse with endosomal structures en route to the plasma membrane (Toomre et al 1999, Polishchuk et al 2000). While PGCs traffic to random sites on the plasma membrane in nonpolarized cells, PGCs containing the membrane protein *mdr1* tagged with GFP in polarized WIF-B cells target uniquely to the bile canaliculus (Sai et al 1999).

Fusion with the Plasma Membrane

Fusion of PGCs with the plasma membrane has been recently studied at the level of single carriers using total internal reflection microscopy (Schmoranzer et al 2000, Toomre et al 2000). High-resolution video rate images were required to analyze fusion-related events occurring at the vicinity of the plasma membrane. The carriers were shown to move out toward the edge of the cell, remain stationary for a variable period (15–30 s), and then rapidly fuse and disperse their fluorescence in the plasma membrane. Fusions were found to occur randomly across the cell surface. These studies also indicated that PGCs fully fuse and integrate all their membrane with the plasma membrane. Future work using total internal reflection microscopy to study exocytosis is likely to help elucidate the role of cytoskeletal and other structural elements involved in fusion and absorption of PGCs into the plasma membrane.

KINETIC ANALYSIS OF SECRETORY TRANSPORT

Quantitative time-lapse imaging data of single cells expressing GFP-tagged cargo molecules have been used to address the kinetic properties of secretory transport, including how long cargo resides in a particular compartment and the rate of cargo influx into and efflux out of a given compartment (Hirschberg et al 1998). Data for this type of analysis were derived from time-lapse images of individual cells expressing VSVG-GFP obtained under conditions where all fluorescent molecules

could be detected at any one time. Changes in fluorescence intensity in the Golgi region and in the entire cell were measured and plotted as VSVG-GFP moved through the secretory pathway after shift to permissive temperature. The changes were then used to address whether a kinetic model with specific rate parameters could fit the data. Remarkably, a simple model consisting of a series of linear rate laws connecting three compartments (ER, Golgi, and plasma membrane) arranged in series was able to fit the data (see Figure 5). More complex, nonlinear rate laws (involving a changing rate constant) were not required even though the concentrations of VSVG-GFP in different compartments went from high (early in the experiment) to low (late in the experiment). This indicates that secretory

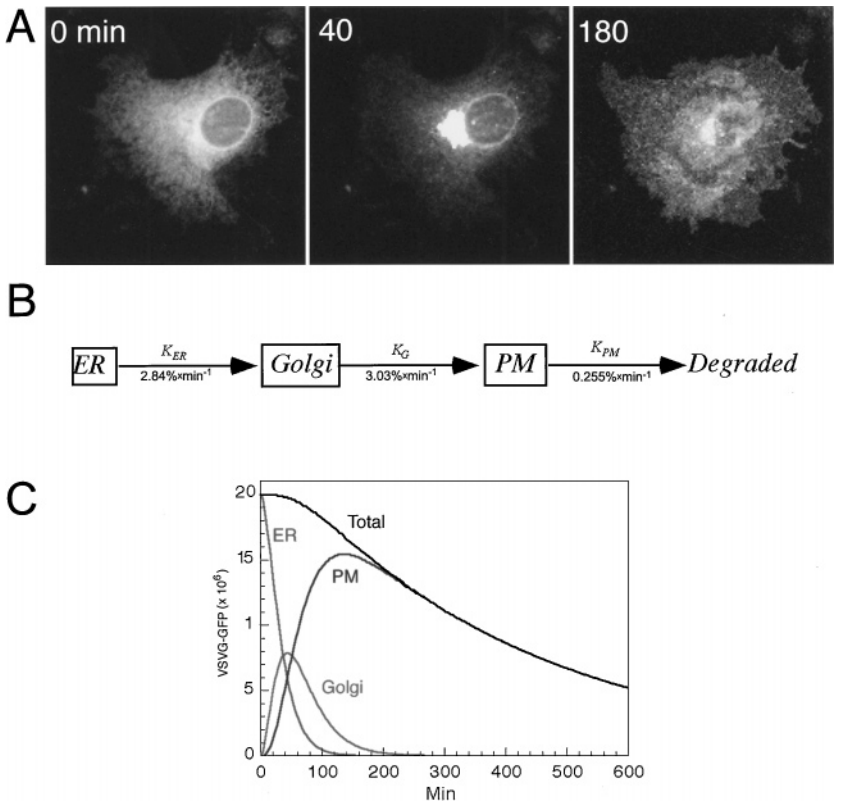

Figure 5 (*A*) Transport of VSVG-GFP through the secretory pathway upon shift from 40 to 32°C. The images show the distribution of VSVG-GFP at 0, 40, and 80 min after temperature shift. (*B*) Compartmental model and rate constants for trafficking of VSVG-GFP that fit the experimental data derived from (*A*) (from Hirschberg et al 1998). (*C*) Changes in levels of VSVG-GFP in ER, Golgi, and plasma membrane [obtained from the trafficking data shown in (*A*)] after VSVG-GFP is released into the secretory pathway upon temperature shift.

transport machinery was never saturated during the experiments and that transport machinery readily accommodates large differences in concentration of cargo. For ER to Golgi transport the mean rate constant was 2.8% per min, for Golgi to plasma membrane transport it was 3.0% per min, and for transport from the plasma membrane to a degradative site it was 0.25%. Interestingly, VSVG-GFP molecules were found arriving at the plasma membrane within 10 min after shift to permissive temperature (see Figure 5). This suggests that there are no lags for VSVG-GFP transport through the Golgi complex, contrary to what might be expected from Golgi cisternal maturation models (Bonfanti et al 1998). The precision in the rate constants estimated from these kinetic modeling experiments was an order of magnitude greater than that obtained in previous biochemical studies because hundreds of data points from the same cell could be collected and analyzed. Biochemical experiments addressing secretory kinetics, by contrast, typically utilize six data points collected from purified fractions of thousands of cells. Future studies using GFP imaging to analyze secretory kinetics are likely to provide insight into the rates at which different molecules traverse the secretory pathway and what steps are subject to pharmacological or physiological control.

SUMMARY AND PERSPECTIVES

The ability to visualize and probe protein trafficking and organelle dynamics using GFP fusion proteins provides a powerful new approach for addressing numerous questions relevant to the regulation and functioning of the secretory pathway. How are proteins retained and sorted within different compartments? What type of protein-protein interactions occur in ER and Golgi membranes? Do different cargo molecules traverse the secretory pathway at different rates and/or in different containers? The many techniques in which GFP chimerae can be used, including time-lapse imaging, quantitation, FRAP, FLIP, fluorescence resonance energy transfer (FRET), double-labeling, and kinetic modeling (reviewed in Lippincott-Schwartz et al 1999, Piston et al 1999), permit analysis of these central questions about protein sorting and trafficking within the secretory pathway. In so doing they offer an important new methodology for relating molecular machinery to the in vivo functioning of secretory membranes.

Future techniques for studying protein trafficking using GFP chimerae will rely on further optimization of GFP expression, continued development of GFP variants with altered spectral properties, and the investigation of the properties of the GFP fluorophore itself (for review see Tsien 1998). As one example, GFPs that are sensitive to pH, including “ecliptic pHluorin” (Miesenböck et al 1998), have been useful in monitoring vesicle exocytosis and recycling because there are changes in pH that accompany release and uptake of vesicle content. A different example is the use of YFP and CFP variants of GFP (Ellenberg et al 1998), which are proving valuable in double-labeling experiments to follow transport of distinct populations of proteins (Keller et al 1998) and in FRET

to study protein-protein interactions at distances between 5 and 10 nm (Miyawaki et al 1997).

Newer GFP-based techniques will also depend on advances in microscopic imaging systems. These include the development of more sensitive and quicker camera systems and superior software for analyzing digital images. The ability to selectively photobleach a specimen, available on some confocal microscope systems, can greatly enhance the imaging of dim structures within cells or in areas of the cell next to very bright objects. Examples include the photobleaching of background fluorescence for visualizing trafficking of GFP-tagged post-Golgi structures (Nakata et al 1998, Hirschberg et al 1998) and selective photobleaching of Golgi or ER regions pools of proteins to measure exchange rates of GFP-tagged proteins between Golgi and ER compartments (Zaal et al 1999). This type of approach is critical for addressing whether small spherical vesicles (50 nm), which are below the resolution of the light microscope but should be detectable if distributed far enough apart (>400 nm), play a significant role in secretory trafficking. Recent advances in microscope imaging techniques, including two-photon laser scanning microscopy (TPLSM) for fluorescence imaging with three-dimensional resolution (Potter et al 1996), total internal reflectance microscopy for studying events at the surface of the coverslip (Schmoranzer et al 2000, Toomre et al 2000), and fluorescence correlation microscopy (Brock & Jovin 1998) for studying protein concentration and diffusion rates, offer additional ways for analyzing the spatial and temporal dynamics of secretory membrane traffic.

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